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A malaria sporozoite surface antigen distinct from the circumsporozoite protein

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Monoclonal antibody NYS4 recognizes a single 140 kDa antigen on the surface of Plasmodium yoelii *sporozoites, an antigen which is distinct from the extensively characterized circumsporozoite (CS) protein. To more thoroughly characterize this additional surface component, a genomic expression library was screened with NYS4 and an immunoreactive clone (M4) was obtained which expressed part of the antigen gene. The deduced amino acid sequence of the M4 peptide included two unique repetitive sequences of amino acids and a conserved sequence motif which is found in several proteins including the CS protein (region II). The cloned DNA hybridized to a PCR (polymerase chain reaction) amplified sporozoite mRNA demonstrating the sporozoite-stage expression of this gene. A synthetic peptide of one of the repeats, (Asn-Pro-Asn-Glu-Pro-Ser), was recognized by NYS4 and mice immunized with (Asn-Pro-Asn-Glu-Pro-Ser)$_3$ conjugated to KLH (keyhole limpet haemocyanin) produced high levels of antibodies that reacted with the surface of sporozoites and specifically to the 140 kDa antigen. Thus, at least two different proteins are on the surface of the* P. yoelii *sporozoite indicating that the immunoreactive exterior of the infective stage of malaria parasites is more antigenically complex than previously thought.*

Introduction

The sporozoite of *Plasmodium* is uniformly covered by a proteinaceous membrane coat which is thought to be composed entirely of the circumsporozoite (CS) protein (*1, 2*). Being the first parasite molecule encountered by the host, the CS protein has been intensively studied and evaluated as a vaccine against malaria. Indeed, experimental immunization with radiation-attenuated sporozoites induces solid protective immunity (*3–5*) coincident with the stimulation of both cellular and humoral immunological responses to the CS protein (*6, 7*). An emerging view is that an essential feature of the protective response induced by sporozoites is cellular immunity (*4–9*). If this is correct, then a multicomponent vaccine containing additional pre-erythrocytic stage antigens may be required to induce sterilizing immunity in all individuals, since those regions of the CS protein that are the immunodominant determinants of T-cell responses are also the most variable in amino acid sequence from parasite to parasite (*10–12*).

The search for additional antigens by Charoenvit et al. led to the production of several monoclonal antibodies that recognize sporozoite determinants which are distinct from the CS protein (*13*). One of these antibodies, designated NYS4 (Navy Yoelii Sporozoite 4), recognizes a sporozoite-specific 140 kDa antigen. This antigen is expressed on the surface of live sporozoites and is secreted along with the CS protein *in vitro* (unpublished results). Wortman et al. used this antibody to isolate an antigen-expressing clone from a *P. yoelii* genomic expression library (*14*). Here we describe the cloning and characterization of a portion of the gene encoding the 140 kDa antigen and identify a hexapeptide of this protein that is a B-cell epitope on sporozoites.

Materials and methods

Parasites and DNA isolation. *P. yoelii* 17 X (NL) parasites were obtained by blood passage in Balb/C mice. DNA isolation from parasite infected blood was performed, as described (*14*).

Genomic expression library and immunoselection. A *P. yoelii* genomic expression library was constructed using 0.5–2.0 kb fragments generated by partial DNase I digestion and commercial (Promega, Madison, WI) lambda gt11 arms and packaging extracts (*15*). The library was screened for antigen expressing clones with a 1:20 dilution of NYS4 (*13*) hybridoma culture supernatant, as described previously (*16–18*). NYS4 immunoreactive plaques were detected with a commercially prepared antibody detection kit

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Fig. 1 (*a*). **Nucleotide sequence and derived amino acid sequence of a portion of the *P. yoelii* genomic DNA insert in lambda gMSY-4 (M4).** The sequences of oligonucleotide primers (+) and (−) used for PCR amplification are indicated.

```
                                                             ------------ (+) ------------>
CCT TTT CTT ACT AAA GTT TGT CAG GAA GTA GAA AGA ATT GCT CAT TGT GGA AAA TGG GAA      60
 P   F   L   T   K   V   C   Q   E   V   E   R   I   A   H   C   G   K   W   E

GAA TGG AGT GAA TGT TCT ACT ACT TGT GAT GAA GGA AGA AAA ATT AGA AGA AGA CAA ATA     120
 E   W   S   E   C   S   T   T   C   D   E   G   R   K   I   R   R   R   Q   I

TTA CAT CCT GGA TGT GTT AGT GAG ATG ACT ACT CCA TGT AAG GTT CGT GAT TGC CCA CAA     180
 L   H   P   G   C   V   S   E   M   T   T   P   C   K   V   R   D   C   P   Q

ATA CCA ATA CCT CCT GTC ATC CCT AAT AAA ATT CCA GAA AAG CCA TCA AAC CCA GAA GAA     240
 I   P   I   P   P   V   I   P   N   K   I   P   E   K   P   S   N   P   E   E
                                    |-----------3-MER REPEATS----------->
CCA GTA AAT CCA AAC GAT CCA AAC GAT|CCA AAC AAC CCA AAC AAC CCA AAT AAC CCA AAC     300
 P   V   N   P   N   D   P   N   D | P   N   N   P   N   N   P   N   N   P   N

AAC CCA AAC AAC CCA AAT AAC CCA AAC AAC CCA AAC AAC CCA AAC AAC CCA AAC AAC CCA     360
 N   P   N   N   P   N   N   P   N   N   P   N   N   P   N   N   P   N   N   P

AAC AAT CCA AAT AAC CCA AAT AAC CCA AAC AAC CCA AAT AAC CCA AAT AAC CCA AAC AAC     420
 N   N   P   N   N   P   N   N   P   N   N   P   N   N   P   N   N   P   N   N
                                        ---------->3-MER REPEATS------------|
CCA AAT AAC CCA AAC AAC CCA AAT AAC CCA AAT AAC CCA AAT AAC CCA AAT AAC|CCA AAC     480
 P   N   N   P   N   N   P   N   N   P   N   N   P   N   N   P   N   N | P   N

GAT CCA TCA AAC CCA AAC AAC CAC CCA AAA AGG CGA AAC CCA AAA AGG CGA AAC CCA AAC     540
 D   P   S   N   P   N   N   H   P   K   R   R   N   P   K   R   R   N   P   N
                                                |-------6-MER REPEAT REGION---->
AAG CCA AAA CCA AAC AAG CCA AAC CCA AAC AAG CCA|AAC CCA AAC GAA CCA TCA AAC CCA     600
 K   P   K   P   N   K   P   N   P   N   K   P | N   P   N   E   P   S   N   P

AAC AAG CCA AAC CCA AAC GAA CCA TCA AAC CCA AAC AAG CCA AAC CCA AAC GAA CCA TCA     660
 N   K   P   N   P   N   E   P   S   N   P   N   K   P   N   P   N   E   P   S

AAC CCA AAC AAG CCA AAC CCA AAT GAG CCA TCA AAC CCA AAC AAG CCA AAC CCA AAT GAG     720
 N   P   N   K   P   N   P   N   E   P   S   N   P   N   K   P   N   P   N   E

CCA TTA AAC CCA AAC GAG CCA TCA AAT CCA AAC GAG CCA TCA AAC CCA AAT GCG CCA TCA     780
 P   L   N   P   N   E   P   S   N   P   N   E   P   S   N   P   N   A   P   S

AAC CCA AAC GAA CCA TCA AAC CCA AAT GAA CCA TCA AAC CCA AAT GAG CCA TCA AAC CCA     840
 N   P   N   E   P   S   N   P   N   E   P   S   N   P   N   E   P   S   N   P

AAC GAA CCA TCA AAC CCA AAT GAA CCA TCA AAC CCA AAA AAG CCA TCA AAC CCA AAT GAG     900
 N   E   P   S   N   P   N   E   P   S   N   P   K   K   P   S   N   P   N   E

CCA TCA AAC CCA AAT GAG CCA TTA AAC CCA AAT GAG CCA TCA AAC CCA AAC GAA CCA TCA     960
 P   S   N   P   N   E   P   L   N   P   N   E   P   S   N   P   N   E   P   S

AAC CCA AAC GAA CCA TCA AAC CCA GAA GAA CCA TCA AAC CCT AAA GAG CCA TCA AAC CCA    1020
 N   P   N   E   P   S   N   P   E   E   P   S   N   P   K   E   P   S   N   P

AAC GAA CCA TCA AAC CCA GAA GAG CCA AAC CCA GAA GAA CCA TCA AAC CCT AAA GAG CCA    1080
 N   E   P   S   N   P   E   E   P   N   P   E   E   P   S   N   P   K   E   P
                                ---------->6-MER REPEAT REGION-----------|
TCA AAC CCA GAA GAG CCA ATA AAC CCA GAA GAA CTA AAC CCA AAA GAG CCA TCA|AAC CCA    1140
 S   N   P   E   E   P   I   N   P   E   E   L   N   P   K   E   P   S | N   P

GAA GAA TCG AAC CCC AAA GAG CCA ATA AAC CCA GAA GAA TCG AAC CCC AAA GAG CCA ATA    1200
 E   E   S   N   P   K   E   P   I   N   P   E   E   S   N   P   K   E   P   I

AAC CCA GAA GAT AAT GAA AAT CCA TTG ATA ATA CAA GAT GAA CCT ATA GAA CCC AGA AAT    1260
 N   P   E   D   N   E   N   P   L   I   I   Q   D   E   P   I   E   P   R   N

GAT TCA AAT GTA ATA CCA ATT TTA CCT ATC ATC CCA CAA AAG GGT AAT AAT ATC CCA AGC    1320
 D   S   N   V   I   P   I   L   P   I   I   P   Q   K   G   N   N   I   P   S
                         <------------(-)------------
AAT CTA CCA GAA AAT CCA TCT GAC TCA GAA GTA GAA TAT CCA AGA CCA AAT GAT AAT GGT    1380
 N   L   P   E   N   P   S   D   S   E   V   E   Y   P   R   P   N   D   N   G

GAA AAT TCA AAT AAT ACT ATG AAA TCA AAA AAA AAT ATA CCC AAC GAG CGG                1431
 E   N   S   N   N   T   M   K   S   K   K   N   I   P   N   E   R
```

(Protoblot, Promega, Madison, WI) according to the manufacturer's specifications.

DNA sequencing. Phage DNA of lambda *gt11* immunoreactive clones was prepared from liquid lysates by standard methods (*19*). Plasmodial DNA insert fragments released by EcoR1 digestion were cloned into M13mp18 (*20*). Overlapping clones spanning the inserts were generated using the M13 RF recombinant plasmid by exonuclease III resection (*21*). Single-stranded templates were sequenced by using Sequenase (United States Biochemical Corp., Cleveland, OH).

Polymerase chain reaction (PCR) amplification of a sporozoite mRNA. RNA from 14-day *P. yoelii* infected mosquitos was purified by the guanidine thiocyanate CsCl gradient technique (*22*) and reverse-transcribed (50 μg whole RNA) by using a commercially available cDNA synthesis kit (Amersham Corp., Arlington Heights, IL) which uses oligo(dT) as primer. One-tenth, one-hundredth, and one-thousandth of the resulting cDNA was subjected to 35 cycles of PCR (*23*) with 2.5 U Taq polymerase (GeneAmp kit, Perkin Elmer Cetus, Norwalk, CT) and 5 μmol each of the following oligonucleotides (Synthecell, Gaithersburg, MD):

(+), 5'-GGGAATTCGCTCATTGTGGAAAATGG-3';
(−), 5'-GGGAATTCTACTTCTGAGTCAGATGG-3'.
Each cycle included denaturation at 94 °C, reannealing at 51 °C, and primer extension at 72 °C.

Peptide synthesis. The peptide (Asn-Pro-Asn-Glu-Pro-Ser)$_3$ was synthesized using a solid-phase method (*24*) on an Applied Biosystems model 430A synthesizer with single coupling of each amino acid. Cleavage and deblocking were performed with 10% trifluoromethanesulfonic acid in trifluoroacetic acid, followed by precipitation in diethyl ether. The peptide was finally redissolved and lyophilized. Analytical HPLC and quantitative amino acid analyses confirmed the identity and purity of the peptide product.

Production of antisera. Six to twelve week old BALB/C mice (Jackson Laboratories, Bar Harbor, ME) were immunized at two-week intervals with 4 doses of 100 μg of (Asn-Pro-Asn-Glu-Pro-Ser)$_3$ conjugated to keyhole limpet haemocyanin. The first dose was emulsified in complete Freund's adjuvant, and subsequent doses in incomplete Freund's adjuvant. Sera were obtained 2 weeks after the fourth dose.

Immunofluorescence and Western blotting. Immunofluorescence and Western blotting were carried out, as previously described (*13*).

Fig. 1 (*b*). **Amino acid sequence comparisons between the M4 peptide (M4, residues 1–48), the CS protein from *P. yoelii* (CSP, residues 282–329 (*29*)), thrombospondin-related anonymous protein from *P. falciparum* (TRAP, residues 229–276 (*27*)), human thrombospondin (residues 405–452 (*25*)), and mouse properdin (residues 39–86 (*26*)).**

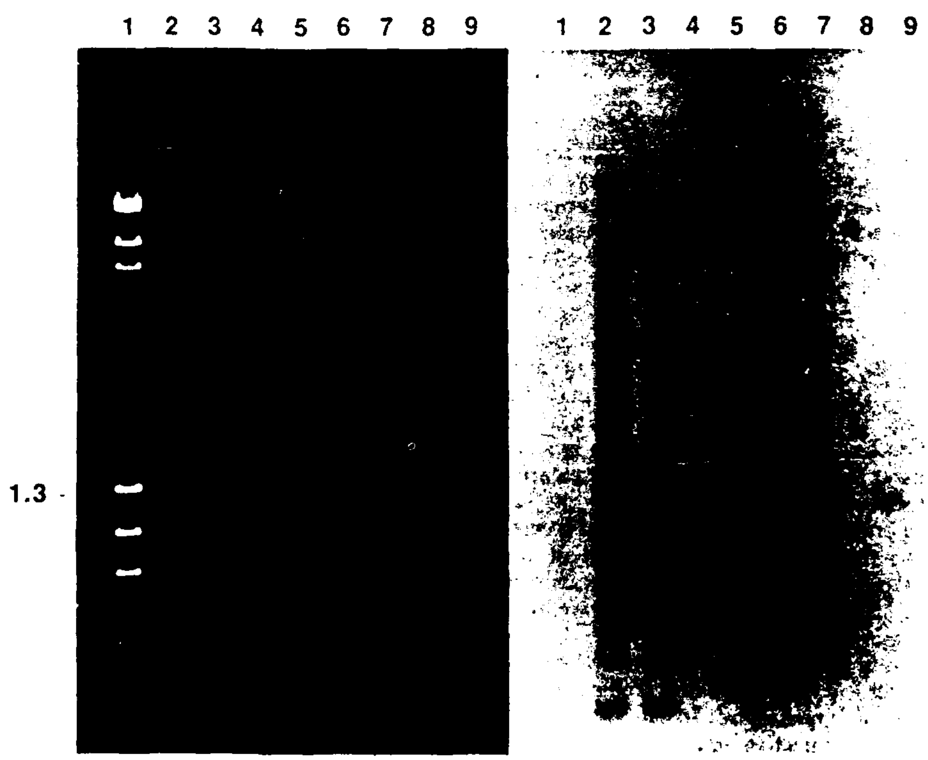
```
M4                    P F L T K V C Q E V E R I A H C G K W E E W S E C S T T C D E G R K I R R R Q I L H P G C V S E
(1-48)
P. yoelii CS protein  V P S A E Q I L E F V K Q I S S Q L T E E W S Q C S V T C G S G V R V R K R K M V K Q P E M L T
(282-329)
P. falciparum TRAP    P F M K A V C V E V E K T A S C G V W D E W S P C S V T C G K G T R S R K R E I L H E G C T S E
(229-276)
thrombospondin        C E I Q E C D K R F K Q D G G W S H W S P W S S C S V T C G D G V I T R I R L C N S P S P Q M N
(405-452)
properdin             E H D G G L C Q - A C R S P Q W S A W S L W G P C S V T C S E G S Q L R H R R C V G R G G Q S E
(39-86)
```

Results

Sequence analysis of the 140 kDa antigen gene. A clone designated lambda gMSY-4 (M4) was selected based on its recognition by NYS4. The primary DNA and deduced amino acid sequences of a portion of the genomic DNA insert from clone M4 are shown in Fig. 1(a). Like most other plasmodial antigens, the M4 peptide contains regions of repeating amino acid sequences. One region consists of a repeating trimer Pro-Asn-Asn and the other is composed of, principally, the hexamer Asn-Pro-Asn-Glu-Pro-Ser. The first 48 amino acids are compared in Fig. 1(b) to several proteins that share a remarkable sequence similarity to this region of the M4 peptide. The similarity between region II of the CS protein (301–315) and thrombospondin is well known (*25*). In addition, mouse properdin was recently shown to contain a similar sequence (*26*). The M4 peptide shares this conserved motif but contains a more extensive region of sequence similarity with the recently described TRAP (thrombospondin-related anonymous protein) from *P. falciparum* (*27*). A search of the PIR protein sequence databank, conducted on 12 February 1990, revealed no additional significant similarity to previously described protein sequences.

mRNA for the 140 kDa antigen is present in sporozoites. To demonstrate sporozoite expression of the mRNA for the 140 kDa antigen we utilized the polymerase chain reaction (PCR) specifically primed with M4 clone sequence-based primers (Fig. 1(b)). An amplification product of the predicted size (the distance between the two primers is 1.3 kB) was clearly visible in reaction mixtures which contained, as templates, sporozoite cDNA or DNA from clone M4 (positive control) (Fig. 2, left). In Fig. 2, right, the identity of the amplified cDNA subfragment was confirmed with the M4 probe. A faint signal at 1.3 kB

Fig. 2. **Expression of the 140 kDa antigen mRNA in sporozoites as determined by PCR amplification of a cDNA subfragment from reverse-transcribed RNA isolated from infected mosquitos.** Primers used in the amplification are shown in Fig. 1. *Left*: agarose gel stained with ethidium bromide. *Right*: Southern blot of the stained agarose gel probed with ^{32}P-labelled M4 clone DNA. Lanes 1, DNA molecular weight markers; lanes 2, M4 clone DNA (15 pg); lanes 3, M4 clone DNA (1.5 pg); lanes 4, cDNA (50 μg); lanes 5, cDNA (5 μg); lanes 6, cDNA (0.5 μg); lanes 7, RNA (50 μg); lanes 8, RNA (5 μg); lanes 9, RNA (0.5 μg).



was detected in control reactions of RNA that were not reverse transcribed prior to PCR. We believe this is due to amplification of the genomic sequence which resulted from DNA contamination of the RNA preparation.

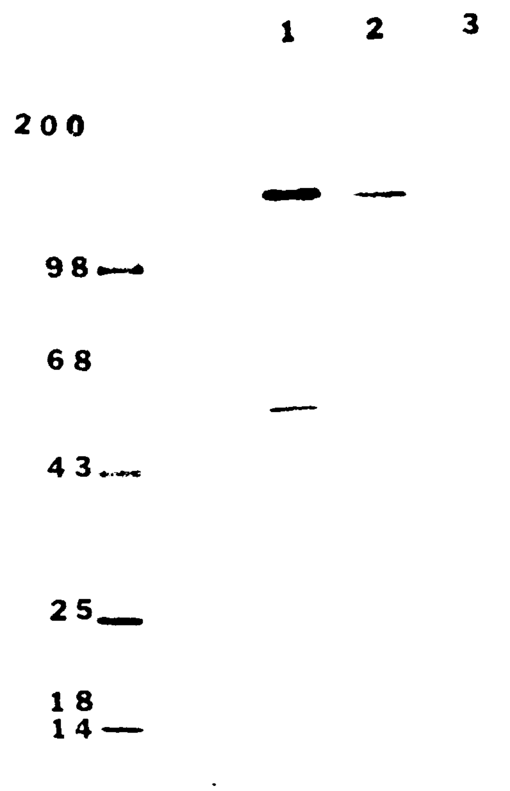
Antibodies to Asn-Pro-Asn-Glu-Pro-Ser recognize the surface of sporozoites and the 140 kDa antigen. The overall hydrophilicity of the repeating amino acid sequence Asn-Pro-Asn-Glu-Pro-Ser suggested it as a potential antigenic determinant (*28*). A synthetic peptide containing 3 copies of Asn-Pro-Asn-Glu-Pro-Ser (18-mer) was strongly recognized by NYS4 in an ELISA (data not shown) and mice immunized with the peptide produced antibodies that reacted with sporozoites in an IFAT and with the 140 kDa antigen on Western blots of sporozoite extracts (Fig. 3). These results together with those of Fig. 2 indicate that the M4 peptide sequence corresponds to at least a portion of the 140 kDa antigen and that the antigenic determinant of NYS4 is contained within the repeating hexamer Asn-Pro-Asn-Glu-Pro-Ser. It is notable that this repetitive sequence bears no similarity to the major repeats of the *P. yoelii* CS protein, in which the consensus repeating elements are Gln-Gly-Pro-Gly-Ala-Pro and Gln-Gln-Pro-Pro (*29*).

Discussion

In this paper we report the molecular characterization of a portion of the gene encoding what we now call sporozoite surface protein 2 (SSP 2). Like the CS protein, SSP 2 contains an immunogenic sequence of repeating amino acids and the conserved region II domain which is found in a number of other well-characterized proteins. These include thrombospondin, properdin, von Willebrand factor, beta$_2$-glycoprotein I, collagen type IV alpha1 & 2 chains, glycoprotein E from human (alpha) herpesvirus 1, and antistasin, all of them proteins which are thought to play roles in adhesion (*30*). The sequence similarity between SSP 2 and TRAP, an erythrocytic-stage specific protein of *P. falciparum*, extends beyond the "adhesion motif" of the CS protein in both the amino terminal and carboxyl terminal directions suggesting a more expansive functional domain in these malarial proteins. Nevertheless the conservation of this sequence among proteins from different malarial species underscores its importance to the survival of the parasite. Perhaps sporozoite entry into host cells is facilitated by SSP 2 adherence to extracellular matrices.

Immunization with irradiation-attenuated sporozoites confers sterile immunity against challenge with large numbers of sporozoites. It has long been thought that this protective immunity is directed entirely against the CS protein. Our findings demonstrate the existence of another protein, SSP 2, on sporozoites that may be an additional target for protective cellular or humoral immune responses.

Fig. 3. **Antibodies to (NPNEPS)$_3$ recognize the 140 kDa sporozoite surface protein.** Western blot of *P. yoelii* sporozoite extract probed with serum from mice immunized with (NPNEPS)$_3$ conjugated to KLH (lane 1), NYS4 (lane 2), and sera from mice immunized with KLH (lane 3).



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